Mammalian target of rapamycin signaling activation patterns in neuroendocrine tumors of the lung.

MAMMALIAN TARGET OF RAPAMYCIN (mTOR) SIGNALLING ACTIVATION PATTERNS IN NEUROENDOCRINE TUMORS OF THE LUNG

Luisella Righi[1], Marco Volante[1] , Ida Rapa[1], Veronica Tavaglione [1], Frediano Inzani[2], Giuseppe Pelosi [3], Mauro Papotti[1].

[1]Divisions of Pathology, Department of Clinical & Biological Sciences, University of Turin at San Luigi Hospital, Orbassano, Torino; [2]Division of Pathology, University of Parma, Parma; [3]Diagnostic Histopathology Unit, European Institute of Oncology and Department of Medicine, Surgery and Dentistry at the University of Milan, Milano, Italy.

Short Title: mTOR signalling pathway in lung neuroendocrine tumors.

Keywords: lung, neuroendocrine tumors, carcinoid, mTOR, signalling.

Address for correspondence:
Luisella Righi, MD
Department of Clinical & Biological Sciences, University of Turin at San Luigi Hospital,
Regione Gonzole 10, 10043 Orbassano, Torino, Italy.
Tel: +390119026018
Fax +390119026753;
e-mail: luisella.righi@unito.it

ABSTRACT

Among alternative therapeutic strategies in clinically aggressive neuroendocrine tumors (NET) of the lung, promising results have been obtained in experimental clinical trials with mTOR inhibitors, though in the absence of a proven mTOR signalling activation status. This study was aimed at analyzing the expression of phosphorylated mTOR (p-mTOR) and of its major downstream activation molecules p70-S6K (p-S6K) and 4EBP1 (p-4EBP1) in a large series of 218 surgically resected, malignant lung NETs, including 24 metastatising typical carcinoids, 73 atypical carcinoids, 60 large cell neuroendocrine carcinomas (LCNEC) and 61 small cell carcinomas (SCLC). By immunohistochemistry, higher levels of p-mTOR and p-S6K were detected in low-to intermediate grade tumors in comparison with high grade tumors ($p<0.001$), at variance with p-4EBP1 which was mainly expressed in LCNEC and SCLC ($p<0.001$). Western blot analysis of NET tumor samples and lung NET cell lines confirmed such findings. A strong correlation between p-mTOR and p-S6K expression ($p<0.0001$) proved the activated status of mTOR pathway. Moreover, p-mTOR protein expression was positively associated with somatostatin receptor(s) expression. None of the investigated molecules impacted on survival. However, in low grade tumors, low p-mTOR expression correlated with lymph node metastases ($p=0.016$), recurrent disease and survival ($p=0.005$). In conclusion, these data demonstrate a differential mTOR activation status in the spectrum of pulmonary NETs, possibly suggesting that mTOR pathway profiling might play a predictive role in patients candidate for mTOR-targeted therapies.

INTRODUCTION

The management of lung neuroendocrine tumors (NET) mainly depends on both grade of differentiation (low-to intermediate *vs* high grade) and clinical stage at diagnosis (localized *vs* metastatic). Surgery is the treatment of choice for low-to intermediate grade (i.e. typical or atypical carcinoids) and localized tumors, while in high grade and/or disseminated lesions chemotherapy is generally preferred (Pelosi *et al.* 2006; Garcia-Yuste *et al.* 2008). Traditional therapies offer limited benefits to patients with advanced disease: the traditional DNA-damaging cytotoxic agents (i.e. platinum-based drugs) have low efficacy and although a large number of therapeutic options have been explored, there is little consensus on a single standard treatment approach (Srirajaskanthan *et al.* 2008), especially in the group of clinically aggressive bronchial carcinoids.

Emerging data on the molecular mechanisms of carcinogenesis and tumor progression prompted a new era of molecular therapeutics with the development of selective targeted agents. In this context, there are several, yet poorly explored, potential therapeutic options for lung NET including somatostatin analogs, inhibitors of the VEGF pathway, and inhibitors of the mammalian Target Of Rapamycin (mTOR) which have shown promising activity in recent clinical studies (Duran *et al.* 2007; Kulke 2007; Yao *et al.* 2008).

mTOR is a serine threonine kinase that participates in the regulation of proliferation, cell growth and apoptosis through modulation of cell cycle progression (Vignot *et al.* 2005). The activated (phosphorylated) mTOR kinase leads to the subsequent phosphorylation of downstream effectors: the ribosomal p70S6-kinase (S6K) and the eukaryotic initiation factor 4E-binding protein 1 (4EBP1), two key proteins that regulate translation of mRNAs into proteins required for cell cycle progression from G1 to S phase (Podsypanina *et al.* 2001; Dancey 2006). Recent insights revealed a significant complexity of the mTOR pathway that seems to cross talk with other well-characterized signalling cascades, thus paving the way to the use of combined therapies (Bjornsti and Houghton 2004; Guertin and Sabatini 2007; Meric-Bernstam and Gonzalez-Angulo 2009). mTOR signalling

pathway can be upstream activated - most commonly via the PI3 Kinase/AKT pathway - by receptors such as somatostatin receptors or insulin-like growth factor receptor 1 or by loss of inhibiting molecules, such as PTEN (von Wichert *et al.* 2000; Wang *et al.* 2002). Rapamycin (Sirolimus, Wyeth, Philadelphia, PA) and its derivates are immunosuppressive macrolides that specifically block mTOR signalling and have been shown to possess anti-proliferative activity in a variety of malignancies both *in vitro* (Zitzmann *et al.* 2007) and in phase II clinical trials (Yao *et al.* 2008). Inhibition of mTOR prevents phosphorylation of S6K, 4EBP1 and, indirectly, other proteins involved in the transcription and cell cycle control, leading to G1 phase cell growth arrest.

Two rapamycin derivates have recently been evaluated in patients with NETs: Temsirolimus (CCI-779; Wyeth, Madison, NJ) and Everolimus (RAD001; Novartis, Basel, Switzerland). A multicentric study has recently demonstrated that Temsirolimus effectively down-regulates the phosphorylation of S6K and that higher baseline levels of phospho-S6K and phospho-mTOR seem to predict a better response in advanced neuroendocrine (NE) carcinomas (Duran *et al.* 2006), although Temsirolimus does not modified the progression-free survival in advanced small cell lung cancer patients (Pandya *et al.* 2007). New perspectives flow from phase I trials aimed at determining the safety, tolerability, pharmacokinetics and pharmacodinamics of novel mTOR inhibitors such as Deforolimus (AP23573, Ariad Pharmaceuticals, Cambridge, MA), that was well tolerated and showed encouraging antitumor activity (Mita *et al.* 2008). Furthermore, *in vitro* studies and *in vivo* clinical trials combining mTOR inhibitors and the somatostatin analog octreotide have recently been published with controversial results in terms of additive anti-tumoral effects of the two compounds (Grozinsky-Glasberg *et al.* 2008; Moreno *et al.* 2008; Yao *et al.* 2008).

Despite all the above pre-clinical and clinical studies on the anti-neoplastic efficacy of mTOR inhibitors in a variety of tumors, data on the activation status of mTOR signalling cascade in pulmonary NET are still lacking. In this respect, a detailed protein expression map of mTOR pathway-related molecules in lung NET, could not only define specific expression patterns predictive of clinical response, as suggested for other malignancies (Lam *et al.* 2007), but also

investigate the prognostic implications of these molecules. Therefore, aim of this study was to evaluate the expression of activated mTOR related proteins in a large series of pulmonary NET - with special reference to clinically malignant cases.

MATERIALS AND METHODS

Case selection. Eight hundred eighty three surgically resected NETs of the lung were recorded between 1989 and 2007 in the pathology files of the Universities of Turin and Parma and the European Institute of Oncology of Milan (467, 188 and 217 cases, respectively). Among them, a series of 218 clinically malignant lesions (129 cases from Turin, 40 from Parma and 49 from Milan) was collected, including 24 typical carcinoids (TC) with lymph node metastases at the time of the diagnosis (TC mets), 73 atypical carcinoids (AC) with or without lymph node metastases, 60 large cell neuroendocrine carcinomas (LCNEC) and 61 small cell carcinomas (SCLC). Pathological samples corresponded to primaries in all but 15 cases, where lymph node metastases were the only available material. In 15 cases, primary tumor and corresponding lymph node metastasis was analyzed. All cases were classified according to the last 2004 WHO Classification on Lung Tumors (Travis *et al.* 2004) and the clinical and pathological characteristics collected elsewere (Righi *et al.* 2009) and including sex, age, primary tumour size, Ki-67 labeling index (expressed as the percentage of positive cells in highest labeling areas), nodal status, stage, follow up and site of distant metastases were detailed in Table 1. Forty consecutive non metastatic TC were raised from the files of the University of Turin to be used as control group for baseline expression of the markers under evaluation. All cases were anonymized by a pathology staff member not involved in the study. Clinical data were compared and analysed through coded data, only. The study was approved by the institutional review board of the Hospital

Immunohistochemistry. Immunohistochemistry was performed using monoclonal antibodies against Ser2448-phospho-mTOR (p-mTOR, rabbit 49F9, diluted 1/100), Thr389-phospho-p70S6K, (p-S6K, mouse 1A5, diluted 1/400) and Thr37/46-phospho-4EBP1 (p-4EBP1, rabbit 236B4, diluted 1/300); all antibodies were purchased from Cell Signalling Technologies, Beverly, MA. Five micron-thick paraffin sections were collected onto charged slides, deparaffinized and re-hydrated in water. After antigen retrieval in pH 6.0 citrate buffer for 5 minutes at 125°C in a pressure cooker, the relevant primary antibodies were incubated overnight at 4°C. Immunoreactions were revealed by a biotin-free dextran-chain detection system (Envision, DakoCytomation, Glostrup, Denmark), and developed using 3'-3'-diaminobenzidine as the chromogen. The specificity of all reactions was validated in parallel control sections omitting the primary antibodies for each immunohistochemical run.

Immunohistochemical data interpretation. Immunohistochemical findings were evaluated independently by two of us (LR and MV) and cases with conflicting scores were reviewed jointly at a multi-head microscope until a consensus was reached. All cases were evaluated using a semi-quantitative histological score (H-score) (Huang *et al.* 2005; Cappia *et al.* 2008) taking into account both the percentage of positive tumor population within the whole section and the immunostaining intensity evaluated subjectively as being negative (0), weak (1), moderate (2) and strong (3). For each case, the H-score was obtained multiplying the percentages of reactive cells by the corresponding immunostaining intensity obtaining a final score ranging from 0 to 300.

Western Blot analysis. Four lung neuroendocrine tumor cell lines (from typical - H727 - and atypical - H720 - carcinoids, and from small cell carcinomas, H69 and H526) were available from ATCC (Manassan, VA, USA). In addition, 14 frozen lung NET samples, not included in the present

series of 218 cases, were available from the tissue bank of the Pathology Unit at the University of Turin.

All samples were homogenized and lysated in TNE lysis buffer supplemented with 1% protease inhibitor cocktail (Complete, Roche Diagnostic Corporation, IN). The protein concentration was evaluated using BCA protein assay Kit (Pierce, Milwaukee, WI), and 50 micrograms of protein were resolved in 8% SDS-PAGE and transferred to nitrocellulose membranes for each experiment. The membrane blots were blocked for 1h with 5% BSA in TBS-Tween 0,1% and incubated overnight 4°C with primary antibody: anti-p-AKT (Thr 308, 1:1000), anti-p-mTOR (Ser2884, 1:1000), anti-p-p70S6K (Thr 389, 1: 2000) (all from Cell Signalling Technology, Beverly, MA). Anti- β-Actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) was used as the loading control. Immunoreactive proteins were visualized using horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibody (1:3,000 and 1:1,000, respectively) and Enhanced Chemioluminescence (ECL) (Amersham, Biosciences, Piscataway, NJ) as the substrate.

Statistical analysis. Statistical analysis was performed using Graphpad 4 software and the results were considered statistically significant at a level of $p<0.05$. One-way ANOVA and non parametric Mann-Whitney *U* tests were used to compare the distribution of the markers investigated among the different tumor groups and with respect to clinical pathological variables. The Spearman test was used to analyze the correlation index among markers expression. Overall survival analysis was performed using the Kaplan-Meier method and Log-Rank test.

RESULTS

Distribution of mTOR signalling molecules in lung NETs. Immunohistochemical staining for p-mTOR and p-4EBP1 provided the expected cytoplasmic pattern, while p-S6K showed either a cytoplasmic perinuclear dot-like or a diffuse nuclear pattern of staining.

In peri-tumoral non neoplastic parenchyma, a weak p-mTOR, p-S6K and p-4EBP1 immunoreactivity was observed in normal bronchial epithelium and endothelia. Alveolar histiocytes were also reactive for p-S6K and p-4EBP1, whereas a strong p-mTOR immunoreactivity was detected in reactive alveolar epithelial cells at the periphery of the tumors (data not shown).

Distribution of p-mTOR and its downstream activation molecules was significantly different among the various NET types (all $p<0.0001$, Figure 1). In particular, p-mTOR and p-S6K were expressed at higher levels in low-to-intermediate grade tumors (LG, corresponding to TC mets and AC) as compared to high-grade carcinomas (HG, corresponding to LCNEC and SCLC) ($p<0.001$ and $p=0.027$, respectively), whereas an opposite distribution held true for p-4EBP1 in LG and HG tumors, respectively ($p<0.001$). In the group of LG tumors, TC mets showed the highest mean H-score values for p-mTOR and p-4EBP1, albeit statistically not different as compared to the control TC and AC groups. At variance, p-S6K H-score distribution was similar in control TC and TC mets, but significantly lower in AC as compared to both TC mets and control TC ($p<0.001$). Notably, a wide dispersion of H-score values was detected within individual tumor groups (range of H-score being 0-220, 0-220, 0-170 and 0-110 for TC mets, AC, LCNEC and SCLC, respectively). In the 15 cases where metastatic tumor tissue was compared to the primary lesion, no significant differences were observed in terms of both the intensity and the percentage of positive cells for any of the three markers under investigation.

Western blot analysis (Figure 2) confirmed the heterogeneity of mTOR pathway activation in lung NETs. Phospho-mTOR and, more markedly, p-AKT and p-S6K proteins were expressed consistently in carcinoid samples, both typical and atypical. Interestingly, the two lung carcinoid cell lines showed opposite activation patterns thus proving to be potential models for functional tests aimed at clarifying the mechanisms of mTOR pathway activation. By contrast, in HG tumor samples both p-mTOR and p-S6K were negative or weakly positive, except for one case. In parallel, p-AKT expression was preserved, though to a generally lower extent, thus suggesting the activation of alternative AKT-mediated signalling pathways in these tumors.

Clinical pathological associations. The distribution of p-mTOR, p-S6K and p-4EBP1 expression according to H-score values and clinical pathological variables is shown in Table 2.

Phospho-mTOR and its downstream effectors did not correlate with proliferation or disease stage. By contrast, in LG tumors high p-mTOR expression associated with parameters indicative of a more favorable outcome, such as negative nodal status (in AC group, p=0.016) and disease free status (p=0.005). Phospho-S6K followed the same association, although slightly below statistical significance, whereas p-4EBP1 did not. Moreover, p-S6K and p-4EBP1 were expressed at higher levels in small tumors, with a strong significance in LG (p-S6K, p=0.006) and HG (p-4EBP1, p=0.008) tumors, respectively.

No association was found between p-mTOR, p-S6K and p-4EBP1 and overall survival, in either LG or HG tumors.

Correlation among mTOR signalling and somatostatin receptor expression. The functional activation of mTOR signalling pathway was defined by analyzing the correlation of expression between p-mTOR and its downstream molecules. A strong positive correlation was observed between p-mTOR and its effector p-S6K. The strong correlation was maintained in LG and HG tumors when analyzed separately. Phospho-4EBP1 weakly correlated with p-mTOR but maintained a significant association with p-S6K (Table 3).

Moreover, data obtained by our group on the expression distribution of somatostatin receptor (SSTR) type 2A and 3 (Righi *et al.* 2009) in the same series, demonstrate an heterogeneous but significant progressive decrease of expression from low to high grade forms. Somatostatin receptor type 2A was over-expressed in metastatic typical carcinoids as compared to atypical carcinoids and clinically benign typical carcinoids. Furthermore a positive correlation was detected between p-mTOR (and p-S6K) and SSTR type 2A. This finding was evident both in LG and HG tumor groups (p=0.034 and p=0.0075, respectively). In addition, although to a lower extent, p-mTOR correlated

to SSTR type 3 expression (p=0.034). By contrast p-4EBP1 did not correlate with SSTRs expression.

DISCUSSION

In the current study, we present the first evidence of activated mTOR signalling pathway in pulmonary NETs, with a specific focus on aggressive forms of these tumors, which are a challenge for the correct clinical management and could benefit from mTOR targeted therapies.

The functional activation of the PI3K/AKT/mTOR signalling pathway has never been extensively investigated in pulmonary or other NETs, except for indirect evidence of the expression of functionally related molecules such as PTEN (Wang *et al.* 2002), tuberous sclerosis complex (TSC) (Yao 2007), AKT (Shah *et al.* 2006) and IGF1R (von Wichert *et al.* 2000).

Nevertheless, the clinical interest in mTOR has increased in recent years, since the development of selective inhibitors, and several preclinical trials have been conducted to test their efficacy in different human malignancies, including NETs (Duran *et al.* 2006; Kulke 2007; Zitzmann *et al.* 2007). However, controversial results in terms of clinical response to mTOR inhibitors have been obtained in NETs, possibly reflecting the heterogeneity of mTOR pathway functional status among different NET entities and within individual tumors of the same histotype.

A growing body of the literature regarding molecular drugs, such as EGFR tyrosine kinase inhibitors, supports the view that the selection of patients is always the fundament to benefit from these therapies at the best. Therefore, the clinical effort on the development of mTOR targeting therapies should be guided by the definition of its pathway activation status within individual NETs, also identifying specific profiles of pathogenetic and predictive interest.

In this context, immunohistochemistry is the most reliable, reproducible, cost-effective and clinically applicable technique to investigate large tumor series, after assuming that specific

antibodies against phosphorylated (active) forms of the target molecules are used through a semi-quantitative evaluation. An example supporting this point of view derives from a phase II trial on the effect of Temsirolimus (a rapamycin derivative) in advanced NE carcinomas. Although this study concluded that this agent had little activity, not warranting further single-agent evaluation in this neoplastic setting, it was clearly shown that Temsirolimus inhibited S6K phosphorylation and that higher baseline levels and lower levels after therapy of p-mTOR were predictive factors of better response (Duran *et al.* 2006).

Our study demonstrates that mTOR is consistently found in pulmonary NETs of different histological types, with a higher expression in low-to-intermediate grade tumors. The correlation between mTOR and its downstream molecules, confirmed also by Western Blot analysis, strongly supports the view that mTOR pathway is functionally activated in a subset of pulmonary NETs. Such correlation was stronger and more significant with p-S6K that is directly involved in mTOR signalling cascade (Guertin and Sabatini 2007) than with p-4EBP1 that, conversely, may be phosphorylated by other kinases, too (Heesom *et al.* 2001; Wang *et al.* 2003). Moreover, the heterogeneous distribution of the molecules under investigation within individual histological subtypes indicates the existence of different functional levels of the pathway, and reinforces the contention that typing different mTOR pathway-related molecules might help to correctly select patients for mTOR inhibitor-guided treatments (Meric-Bernstam and Gonzalez-Angulo 2009). In this respect, a weakness of the present study is its retrospective character and the lack of clinical correlates between mTOR and related molecule(s) expression, and the clinical response of patients to mTOR inhibitor treatments. This limitation partly reflects the current lack of standardized therapeutic approaches to the use of these drugs in the setting of clinically aggressive lung NETs.

Another interesting finding is the correlation that mTOR demonstrated with SSTR expression of both 2A and 3 types. Biologically, this observation seems to support the view that mTOR activity might be modulated also by SSTR in the light of experimental observations on octreotide capability to down-regulate mTOR-upstream molecules, such as PI3 Kinase and AKT, eventually leading to

anti-proliferative activity (Theodoropoulou *et al.* 2006). Such cross-talk between SSTRs and mTOR might explain the results of recent *in vitro* and *in vivo* studies on the anti-tumoral efficacy of combined mTOR inhibitor and octreotide treatment in NETs (Grozinsky-Glasberg *et al.* 2008; Moreno *et al.* 2008; Yao *et al.* 2008).

In the current tumor series, all the mTOR-related molecules failed to show a significant impact on overall survival. However, higher levels of p-mTOR and p-S6K expression were associated to more favorable clinico-pathological parameters; for example, they were found in LG tumor groups and associated with negative nodal status (in the AC group, only) or with disease free status (in LG tumor group). By contrast, 4EBP1 was unrelated to clinical pathological characteristics, suggesting activation from alternative kinase pathways other than mTOR. The literature on the prognostic role of mTOR pathway activation status in human tumors is scanty and controversial, supporting a favorable impact in some models, such as ovarian cancer (Noske *et al.* 2008), and an adverse prognostic effect in others, such as renal cell (Pantuck *et al.* 2007; Campbell *et al.* 2008), breast (Noh *et al.* 2008) and biliary tract (Herberger *et al.* 2007) carcinomas. However, no study was previously designed to investigate the prognostic implications of mTOR pathway activation players in NETs.

In conclusion, we first described the activation pattern of mTOR/S6K/4EBP1 signalling pathway in a large series of aggressive pulmonary NETs, also providing evidence for cross-talking with the SSTR pathway. These data support the concept that a detailed protein mapping of mTOR pathway-related molecules in lung (and possibly other) NETs may drive a more selective strategy for targeting mTOR in individual neuroendocrine tumors.

Declaration of interest: all Authors declare the absence of any conflict of interest.

Financial support: Work supported by grants from the Italian Ministry of University (ex 60% to MV and MP) and from un-restricted grants from Novartis Pharma to MV (FT75/08) and MP (FT81/07).

Acknowledgements: We are grateful to Professors G. Bussolati and F. Ardissone (University of Turin) and to Professors G. Rindi and M. Rusca (University of Parma) for their helpful suggestions.

REFERENCES

Bjornsti MA & Houghton PJ 2004 The TOR pathway: a target for cancer therapy. *Nat Rev Cancer* 4 335-348.

Campbell L, Jasani B, Edwards K, Gumbleton M & Griffiths DF 2008 Combined expression of caveolin-1 and an activated AKT/mTOR pathway predicts reduced disease-free survival in clinically confined renal cell carcinoma. *Br J Cancer* 98 931-940.

Cappia S, Righi L, Mirabelli D, Ceppi P, Bacillo E, Ardissone F, Molinaro L, Scagliotti GV & Papotti M 2008 Prognostic role of osteopontin expression in malignant pleural mesothelioma. *Am J Clin Pathol* 130 58-64.

Dancey JE 2006 Therapeutic targets: MTOR and related pathways. *Cancer Biol Ther* 5 1065-1073.

Duran I, Kortmansky J, Singh D, Hirte H, Kocha W, Goss G, Le L, Oza A, Nicklee T, Ho J et al. 2006 A phase II clinical and pharmacodynamic study of temsirolimus in advanced neuroendocrine carcinomas. *Br J Cancer* 95 1148-1154.

Duran I, Salazar R, Casanovas O, Arrazubi V, Vilar E, Siu LL, Yao J & Tabernero J 2007 New drug development in digestive neuroendocrine tumors. *Ann Oncol* 18 1307-1313.

Garcia-Yuste M, Matilla JM & Gonzalez-Aragoneses F 2008 Neuroendocrine tumors of the lung. *Curr Opin Oncol* 20 148-154.

Grozinsky-Glasberg S, Franchi G, Teng M, Leontiou CA, Ribeiro de Oliveira A, Jr., Dalino P, Salahuddin N, Korbonits M & Grossman AB 2008 Octreotide and the mTOR inhibitor RAD001 (everolimus) block proliferation and interact with the Akt-mTOR-p70S6K pathway in a neuro-endocrine tumour cell Line. *Neuroendocrinology* 87 168-181.

Guertin DA & Sabatini DM 2007 Defining the role of mTOR in cancer. *Cancer Cell* 12 9-22.

Heesom KJ, Gampel A, Mellor H & Denton RM 2001 Cell cycle-dependent phosphorylation of the translational repressor eIF-4E binding protein-1 (4E-BP1). *Curr Biol* 11 1374-1379.

Herberger B, Puhalla H, Lehnert M, Wrba F, Novak S, Brandstetter A, Gruenberger B, Gruenberger T, Pirker R & Filipits M 2007 Activated mammalian target of rapamycin is an adverse prognostic factor in patients with biliary tract adenocarcinoma. *Clin Cancer Res* 13 4795-4799.

Huang WC, Xie Z, Konaka H, Sodek J, Zhau HE & Chung LW 2005 Human osteocalcin and bone sialoprotein mediating osteomimicry of prostate cancer cells: role of cAMP-dependent protein kinase A signaling pathway. *Cancer Res* 65 2303-2313.

Kulke MH 2007 New developments in the treatment of gastrointestinal neuroendocrine tumors. *Curr Oncol Rep* 9 177-183.

Lam JS, Pantuck AJ, Belldegrun AS & Figlin RA 2007 Protein expression profiles in renal cell carcinoma: staging, prognosis, and patient selection for clinical trials. *Clin Cancer Res* 13 703s-708s.

Meric-Bernstam F & Gonzalez-Angulo AM 2009 Targeting the mTOR signaling network for cancer therapy. *J Clin Oncol* 27 2278-2287.

Mita MM, Mita AC, Chu QS, Rowinsky EK, Fetterly GJ, Goldston M, Patnaik A, Mathews L, Ricart AD, Mays T et al. 2008 Phase I trial of the novel mammalian target of rapamycin inhibitor deforolimus (AP23573; MK-8669) administered intravenously daily for 5 days every 2 weeks to patients with advanced malignancies. *J Clin Oncol* 26 361-367.

Moreno A, Akcakanat A, Munsell MF, Soni A, Yao JC & Meric-Bernstam F 2008 Antitumor activity of rapamycin and octreotide as single agents or in combination in neuroendocrine tumors. *Endocr Relat Cancer* 15 257-266.

Noh WC, Kim YH, Kim MS, Koh JS, Kim HA, Moon NM & Paik NS 2008 Activation of the mTOR signaling pathway in breast cancer and its correlation with the clinicopathologic variables. *Breast Cancer Res Treat* 110 477-483.

Noske A, Lindenberg JL, Darb-Esfahani S, Weichert W, Buckendahl AC, Roske A, Sehouli J, Dietel M & Denkert C 2008 Activation of mTOR in a subgroup of ovarian carcinomas: correlation with p-eIF-4E and prognosis. *Oncol Rep* 20 1409-1417.

Pandya KJ, Dahlberg S, Hidalgo M, Cohen RB, Lee MW, Schiller JH & Johnson DH 2007 A randomized, phase II trial of two dose levels of temsirolimus (CCI-779) in patients with extensive-stage small-cell lung cancer who have responding or stable disease after induction chemotherapy: a trial of the Eastern Cooperative Oncology Group (E1500). *J Thorac Oncol* 2 1036-1041.

Pantuck AJ, Seligson DB, Klatte T, Yu H, Leppert JT, Moore L, O'Toole T, Gibbons J, Belldegrun AS & Figlin RA 2007 Prognostic relevance of the mTOR pathway in renal cell carcinoma: implications for molecular patient selection for targeted therapy. *Cancer* 109 2257-2267.

Pelosi G, Volante M, Papotti M, Sonzogni A, Masullo M & Viale G 2006 Peptide receptors in neuroendocrine tumors of the lung as potential tools for radionuclide diagnosis and therapy. *Q J Nucl Med Mol Imaging* 50 272-287.

Podsypanina K, Lee RT, Politis C, Hennessy I, Crane A, Puc J, Neshat M, Wang H, Yang L, Gibbons J et al. 2001 An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten+/- mice. *Proc Natl Acad Sci U S A* 98 10320-10325.

Righi L, Volante M, Tavaglione V, Billè A, Daniele L, Angusti T, Inzani F, Pelosi G, Rindi G & Papotti M 2009 Somatostatin Receptor tissue distribution in lung neuroendocrine tumours: a clinicopathologic and immunohistochemical study of 218 “clinically aggressive” cases. *Annals of Oncology* in press.

Shah T, Hochhauser D, Frow R, Quaglia A, Dhillon AP & Caplin ME 2006 Epidermal growth factor receptor expression and activation in neuroendocrine tumours. *J Neuroendocrinol* 18 355-360.

Srirajaskanthan R, Toumpanakis C, Karpathakis A, Marelli L, Quigley AM, Dusmet M, Meyer T & Caplin ME 2008 Surgical management and palliative treatment in bronchial neuroendocrine tumours: A clinical study of 45 patients. *Lung Cancer*.

Theodoropoulou M, Zhang J, Laupheimer S, Paez-Pereda M, Erneux C, Florio T, Pagotto U & Stalla GK 2006 Octreotide, a somatostatin analogue, mediates its antiproliferative action in pituitary tumor cells by altering phosphatidylinositol 3-kinase signaling and inducing Zac1 expression. *Cancer Res* 66 1576-1582.

Travis WD, Brambilla E, Muller-Hermelink HK & Harris CC 2004 *Tumours of the Lung, Pleura, Thymus and Heart*. Lyon: IARC Press.

Vignot S, Faivre S, Aguirre D & Raymond E 2005 mTOR-targeted therapy of cancer with rapamycin derivatives. *Ann Oncol* 16 525-537.

von Wichert G, Jehle PM, Hoeflich A, Koschnick S, Dralle H, Wolf E, Wiedenmann B, Boehm BO, Adler G & Seufferlein T 2000 Insulin-like growth factor-I is an autocrine regulator of chromogranin A secretion and growth in human neuroendocrine tumor cells. *Cancer Res* 60 4573-4581.

Wang L, Ignat A & Axiotis CA 2002 Differential expression of the PTEN tumor suppressor protein in fetal and adult neuroendocrine tissues and tumors: progressive loss of PTEN expression in poorly differentiated neuroendocrine neoplasms. *Appl Immunohistochem Mol Morphol* 10 139-146.

Wang X, Li W, Parra JL, Beugnet A & Proud CG 2003 The C terminus of initiation factor 4E-binding protein 1 contains multiple regulatory features that influence its function and phosphorylation. *Mol Cell Biol* 23 1546-1557.

Yao JC 2007 Neuroendocrine tumors. Molecular targeted therapy for carcinoid and islet-cell carcinoma. *Best Pract Res Clin Endocrinol Metab* 21 163-172.

Yao JC, Phan AT, Chang DZ, Wolff RA, Hess K, Gupta S, Jacobs C, Mares JE, Landgraf AN, Rashid A et al. 2008 Efficacy of RAD001 (everolimus) and octreotide LAR in advanced low-

to intermediate-grade neuroendocrine tumors: results of a phase II study. *J Clin Oncol* 26 4311-4318.

Zitzmann K, De Toni EN, Brand S, Goke B, Meinecke J, Spottl G, Meyer HH & Auernhammer CJ 2007 The novel mTOR inhibitor RAD001 (everolimus) induces antiproliferative effects in human pancreatic neuroendocrine tumor cells. *Neuroendocrinology* 85 54-60.

FIGURE LEGENDS

Figure 1. Immunohistochemical distribution of p-mTOR, p-S6K and p-4EBP1 in lung NETs. Upper panels illustrate an intense p-mTOR and p-S6K as well as a weak p-4EBP1 expression in a case of TC mets as compared to a case of LCNEC (middle panels) showing the opposite features. In lower panels the histograms of mTOR related molecules distribution are represented (with levels of significance for overall differences in black, for LG as compared to HG tumors in red, and for individual groups as compared to control TC at the top of each column; ns: not significant; *: $p<0.01$; **: $p<0.001$). Abbreviations: TC mets: typical carcinoids with lymph node metastases; AC: atypical carcinoids; LCNEC: large cell neuroendocrine carcinoma; SCLC: small cell lung carcinoma; LG tumors: low grade tumors, including typical carcinoids with metastases and atypical carcinoids; HG tumors: high grade carcinomas, including large and small cell neuroendocrine carcinomas.

Figure 2: Western blot analysis of 15 cases of lung NETs and 4 lung NET cell lines.
Abbreviations: TC: typical carcinoid; AC: atypical carcinoid; LCNEC: large cell neuroendocrine carcinoma; SCLC: small cell lung carcinoma; LG: low grade; HG: high grade carcinomas.

Table 1. Clinicopathologic features of 218 aggressive pulmonary neuroendocrine tumors (Righi et al, 2009).

	TC mets (#24) *No.a.*		AC (#73) *No.a.*		LCNEC (#60) *No.a.*		SCLC (#61) *No.a.*	
Sex	24		73		60		61	
M		12		42		53		49
F		12		31		7		12
Age (y)	24		73		60		61	
Range		15-78		11-77		35-87		44-84
Mean		48		55		64		65
Median		49		57		65		66
Primary tumour size (mm)	23		62		51		47	
≤10		1		6		2		0
11-29		14		24		16		15
≥30		8		32		33		32
Mean		25		32		42		38
Ki67 (%)	18		49		39		44	
Mean		3		16		70		76
Range		0,3-8		1-70		30-90		40-95
Nodal status	23		61		49		57	
N0		0		32		30		22
N1		16		16		11		14
N2-3		7		13		8		21
Stage	23		57		50		51	
1A-1B		0		13-17		9-14		9-11
2A-2B		9-6		6-8		1-12		3-9
3A-3B		8-0		9-4		7-3		15-2
4		0		0		4		2
Follow-up (mos)	24		72		58		61	
Follow up time:								
range		2-175		1-215		1-214		1-120
mean		47		57		41		27
Mean OS		*n.r*		122		30		23
Disease status	24		72		58		61	
NED/DOC		22		47		20		21
AWD		2		5		0		0
DOD		0		20		38		40
Site of distant metastases		Liv: 1 Lu: 1		Adr: 1 BM: 1 Bo: 3 CNS: 1 Liv: 5 Lu: 1 Med: 2 Ov:1 Pc:1 Thy:1		Bo: 4 ChW: 1 CNS: 1 Liv: 3 Lu: 3		Adr: 1 AxLN:1 Bo: 6 ChW: 1 CNS: 7 Liv: 9 Lu: 6 Med: 3

Abbreviations: TC mets: typical carcinoid with metastases; AC: atypical carcinoid; LCNEC: large cell neuroendocrine carcinoma; SCLC: small cell lung carcinoma; No.a.= number of available cases; M: male; F: female; RUL: right upper lobe; RML: right medium lobe; RIL: right inferior lobe; LUL: left upper lobe; LIL: left inferior lobe; n.a= not applicable; mos= months; NED: not evidence of disease; DOC: death of other causes; AWD: alive with disease; DOD: death of disease; OS: overall survival; nr: not reached; Adr: adrenal gland; AxLN: axillary lymph node; BM: bone marrow; Bo: bone; ChW: chest wall; CNS: central nervous system; Liv: liver; Lu: lung; Med: mediastinum; Ov: ovary; Pc: pancreas; Thy: thyroid.

Table 2. Distribution of the p-mTOR, p-S6K and p-4EBP1 expression levels according to clinical and pathological variables in 218 aggressive pulmonary neuroendocrine tumors.

	p-mTOR (mean H-s)	*p*	p-S6K (mean H-s)	*p*	p-4EBP1 (mean H-s)	*p*
Size (mm)						
LG tumors (#97)						
≤30	68.5	0.204	63.6	**0.006**	87.2	0.06
>30	56.3		29.7		52.8	
HG carcinomas (#121)						
≤30	26.5	0.53	33.7	0.22	139.3	**0.008**
>30	23.1		19.3		95.8	
Ki-67*						
TCmets (#18)						
≤2	71.5		96	0.89	74	0.63
>2	72.5		96.3		107.5	
AC (#49)		0.89				
≤10	65.7	0.2	40	0.64	72.8	0.19
>10	30.2		29.5		52.6	
HG carcinomas (#83)		0.59				
≤75	13.7		24.9	0.23	102.5	0.36
>75	15.1		28.5		118.3	
Nodal status						
AC (#61)						
N0	81.2	**0.016**	48.44	0.09	77.0	0.33
N+	33.4		23.45		53.8	
HG carcinomas (#106)						
N0	25.4	0.62	27.8	0.25	112.8	0.97
N+	17.4		19.7		114.8	
Clinical Stage (TNM 2002)						
LG tumors (#96)						
Stages 1-2	62.5	0.80	44.6	0.75	64.8	0.96
Stages 3-4	61.2		46.7		64.3	
HG carcinomas (#101)						
Stages 1-2	21.3	0.29	23.8	0.82	115.7	0.89
Stages 3-4	24.1		21.7		114.2	
Vital status						
LG tumors (#96)						
NED/DOC	71.9	**0.005**	53.6	0.06	75	0.96
AWD/DOD	31		40		83	
HG carcinomas (#119)						
NED/DOC	21.7	0.89	28.7	0.49	122.6	0.48
AWD/DOD	21.9		24.9		111.8	

Mean Survival (months)						
LG tumors (#96)						
low	nr	0.63	104	0.152	nr	0.81
high	122		nr		122	
HG carcinomas (#119)						
low	25	0.44	34	0.258	28	0.63
high	28		23		27	

Abbreviations: H-s: H-score; TC mets: typical carcinoids with metastases; AC: atypical carcinoids; LG tumors: low grade tumors, including typical carcinoids with metastases and atypical carcinoids; HG carcinomas: high grade carcinomas, including large and small cell neuroendocrine carcinomas; *: cut off values of Ki-67 correspond to medians in each group; NED: not evidence of disease; DOC: death for other causes; AWD: alive with disease; DOD: death of disease; nr: not reached.

Table 3. Correlation of p-mTOR, p-S6K and 4EBP-1 with somatostatin receptors in 218 pulmonary neuroendocrine tumors.

	p-mTOR	**p-S6K**	**p-4EBP1**
p-mTOR	-	**p<0.0001** ***r=0.458***	**p=0.0074** ***r=0.181***
p-S6K	-	-	**p<0.0001** ***r=0.312***
SSTR-2A	**p<0.0001** ***r=0.271***	**p=0.046** ***r=0.135***	p=0.7 *r=-0.023*
SSTR-3	**p=0.034** ***r=0.271***	p=0.9 *r=-0.069*	p=0.9 *r=0.016*

Abbreviations: SSTR: somatostatin receptor.

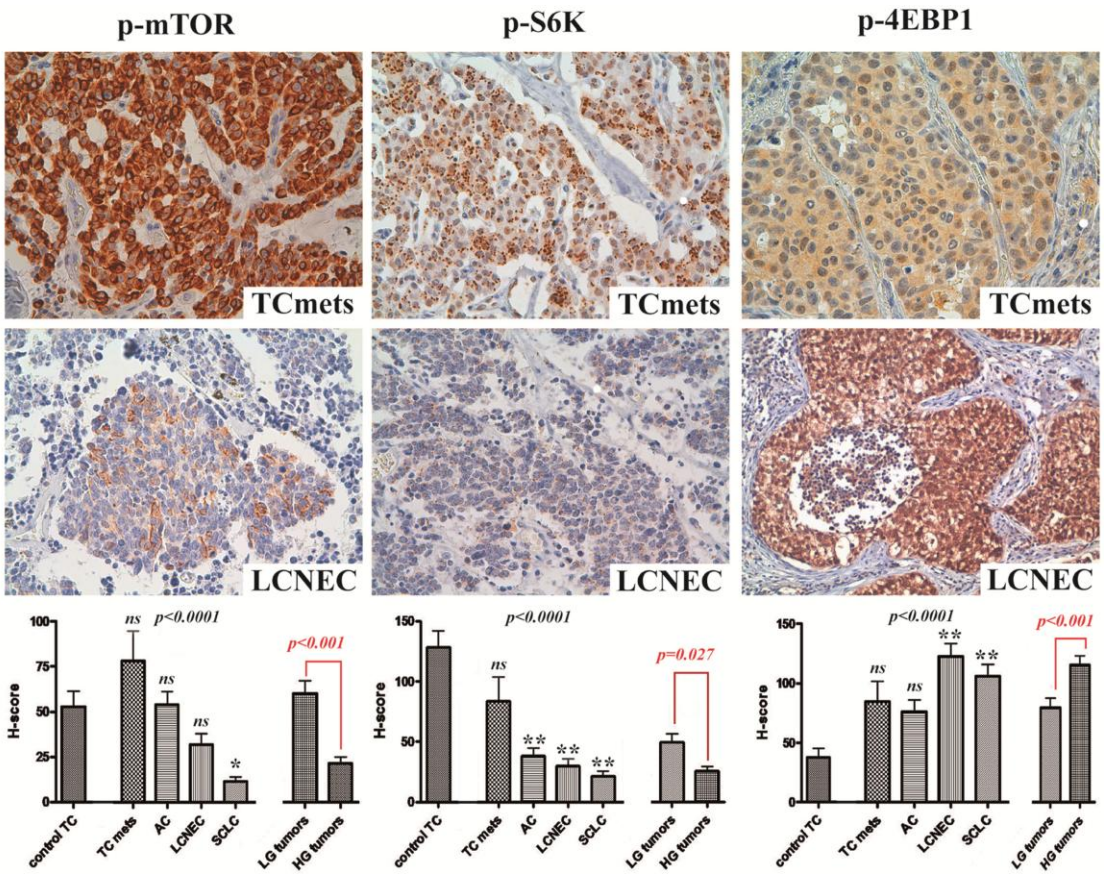
1
p-mTOR
p-S6K
p-4EBP1
TCmets
TCmets
TCmets
LCNEC
LCNEC
LCNEC
H-score
ns
p<0.0001
ns
ns
*
p<0.001
control TC
TC mets
AC
LCNEC
SCLC
LG tumors
HG tumors
p<0.0001
ns
**
**
**
p=0.027
p<0.0001
ns
ns
**
**
p<0.001

Figure 1

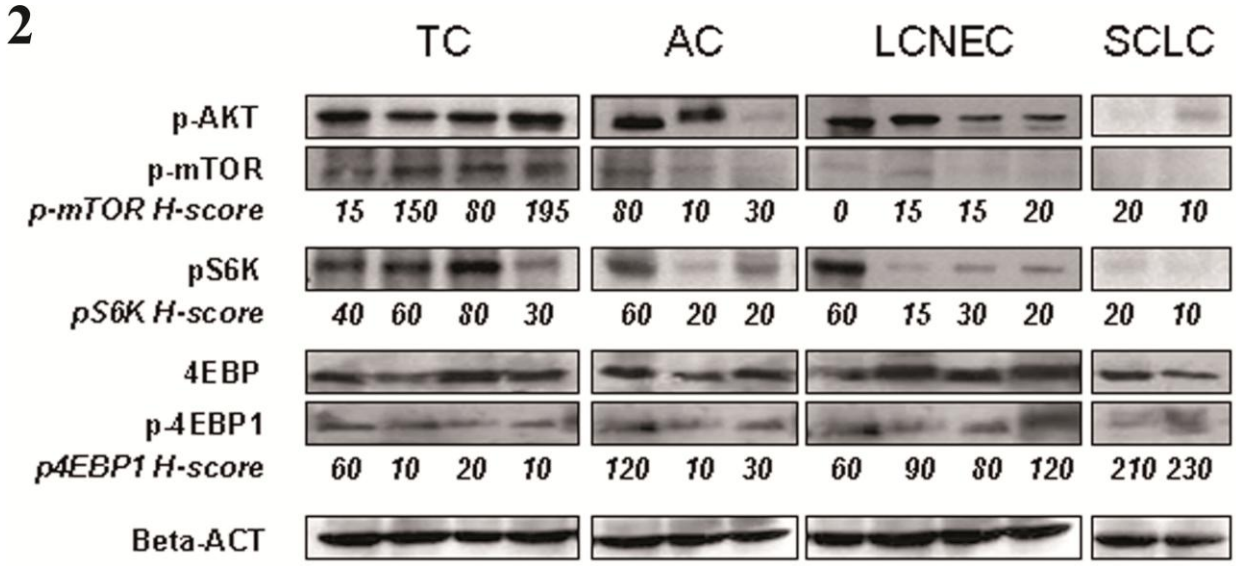
2
TC
AC
LCNEC
SCLC
p-AKT
p-mTOR
p-mTOR H-score
15 150 80 195 80 10 30 0 15 15 20 20 10
pS6K
pS6K H-score
40 60 80 30 60 20 20 60 15 30 20 20 10
4EBP
p-4EBP1
p4EBP1 H-score
60 10 20 10 120 10 30 60 90 80 120 210 230
Beta-ACT

Figure 2

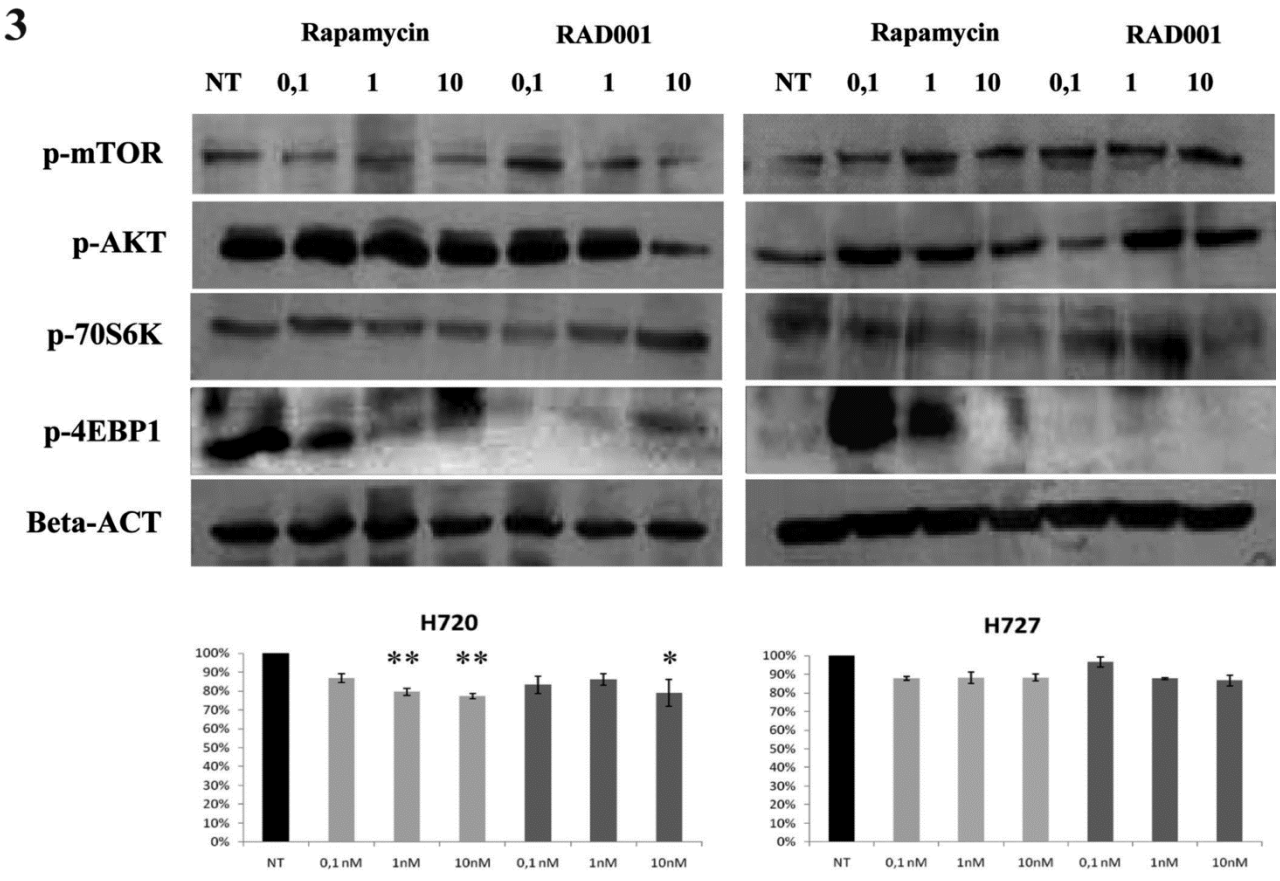
3
Rapamycin
RAD001
Rapamycin
RAD001
NT 0,1 1 10 0,1 1 10
NT 0,1 1 10 0,1 1 10
p-mTOR
p-AKT
p-70S6K
p-4EBP1
Beta-ACT
H720
H727
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
**
**
*
NT
0,1 nM
1nM
10nM
0,1 nM
1nM
10nM

Figure 3